Calcium-sensing receptor antagonism or lithium treatment ameliorates aminoglycoside-induced cell death in renal epithelial cells

Claire E. Gibbons [a],[1], David Maldonado-Pérez [b],[c],[1], Amish N. Shah [a],
Daniela Riccardi [b], Donald T. Ward [a],*

[a] *Faculty of Life Sciences, The University of Manchester, Manchester, UK*
[b] *Cardiff School of Biosciences, Cardiff University, Cardiff, UK*
[c] *MRC Human Reproductive Sciences Unit, Edinburgh, UK*

Abstract

The aminoglycoside antibiotic gentamicin elicits proximal tubular toxicity and cell death. In calcium-sensing receptor (CaR)-transfected HEK-293 (CaR-HEK) cells and CaR-expressing proximal tubule-derived opossum kidney (OK) cells, chronic gentamicin treatment elicits dose-dependent, caspase-mediated apoptotic cell death. Here we investigated whether the renal cell toxicity of the CaR agonist gentamicin could be prevented by CaR antagonism or by lithium cotreatment which may interfere with receptor-mediated signalling. Chronic treatment of OK and CaR-HEK cells with low concentrations of gentamicin elicited cell death, an effect that was ameliorated by cotreatment with the CaR negative allosteric modulator (calcilytic) NPS-89636. This calcilytic also attenuated CaR agonist-induced ERK activation in these cells. In addition, 1 mM LiCl, equivalent to its therapeutic plasma concentration, also inhibited gentamicin-induced toxicity in both cell types. This protective effect of lithium was not due to the disruption of phosphatidylinositol-mediated gentamicin uptake as the cellular entry of Texas red-conjugated gentamicin into OK and CaR-HEK cells was unchanged by lithium treatment. However, the protective effect of lithium was mimicked by glycogen synthase 3β inhibition. Together, these data implicate CaR activation and a lithium-inhibitable signalling pathway in the induction of cell death by gentamicin in renal epithelial cells in culture.
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1. Introduction

The aminoglycoside antibiotics (AGAs) are an effective and economical treatment for life-threatening, Gram-negative infections. However, the clinical usefulness of the AGAs is limited by their toxicity in the renal proximal tubule as well as their ototoxicity [1]. We have shown previously that proximal tubule-derived opossum kidney (OK) cells express a calcium-sensing receptor (CaR)-like protein and elicit typical responses to CaR agonists [2]. More recently we have shown that chro-

nic exposure of OK cells to gentamicin, or indeed other CaR agonists such as spermine or poly-arginine, promotes cell death [3]. In addition, the effect of gentamicin on cell-fate was also investigated using HEK-293 cells and it was found that cells transfected with the CaR were considerably more susceptible to AGA toxicity than non-transfected or empty vector-transfected controls. Thus, given that CaR is expressed at the apical surface of the proximal tubule [4,5], where gentamicin-induced nephrotoxicity occurs, and where the acidic conditions enhance the potency of gentamicin as a CaR agonist [6], these cell culture data suggest that the CaR could contribute to AGA nephrotoxicity. However, in the absence of pharmacological inhibitors of the receptor it has not been previously possible to confirm an involvement of CaR in AGA-mediated cellular toxicity. Furthermore, the concentrations of gentamicin employed in the previous cell culture studies were at least an order of magnitude

* Corresponding author. Faculty of Life Sciences, Floor 2, Core Technology Facility, The University of Manchester, 46 Grafton Street, Manchester, M13 9NT, United Kingdom. Tel.: +44 161 275 5459; fax: +44 161 275 5600.
E-mail address: d.ward@manchester.ac.uk (D.T. Ward).
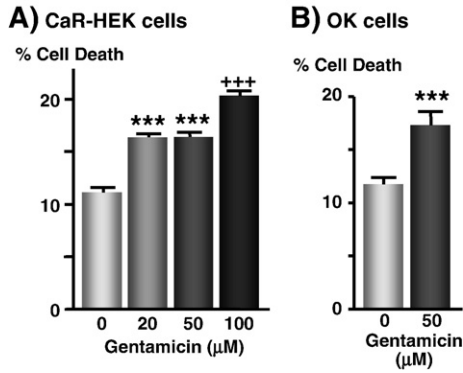[1] These authors contributed equally.

Fig. 1. Gentamicin induces cell death in CaR-HEK and OK cells at low concentrations. CaR-HEK cells were treated for 6 days with 20, 50 or 100 μM gentamicin, with addition of fresh serum (1%) to each dish at Day 3. Cell death was then determined by trypan blue exclusion (panel A, $N=7$–8) including both floating and adherent cells. Alternatively, OK cells were treated for 7 days with 50 μM gentamicin, with addition of fresh serum (1%) to each dish at Day 3 and then cell death quantified as before (panel B, $N=12$). ***$P<0.001$ vs control; +++$P<0.001$ vs all others by ANOVA.

higher than those used clinically [1] and therefore the contribution of CaR to AGA toxicity in renal cell culture could not be demonstrated directly. Therefore, here we began by assessing the contribution of the CaR to AGA renal cell toxicity by testing whether a negative allosteric modulator of CaR, the calcilytic NPS-89636, exerted a protective effect in renal-derived cells treated chronically with therapeutically relevant concentrations of gentamicin.

Despite being first described as a mood stabilizer in 1949 [7], lithium currently remains an effective and inexpensive treatment for bipolar disorder [8]. Its therapeutic mechanism of action may result from its interference in phosphatidylinositol turnover [9] given the inhibitory effect of lithium on inositol monophosphatase [10,11]. However, more recently the ability of lithium to inhibit glycogen synthase kinase (GSK)-3β [12] has been one of a number of mechanisms postulated to explain its therapeutic effect [13]. In the context of AGA nephrotoxicity, it has been reported that in rats injected with gentamicin for 5 days, concurrent therapy with lithium decreased the gentamicin-induced formation of renal lysosomal myeloid bodies [14], possibly via interference of lithium with phosphoinositide turnover. As a hydrophilic, polycationic drug, gentamicin binds negatively-charged phospholipid bilayers and as such this binding could contribute to the cellular uptake of gentamicin, at least in association with the binding of the drug to the megalin / cubilin complex [15,16]. Therefore, we then tested whether lithium could ameliorate gentamicin-induced toxicity in OK and CaR-HEK cells, and whether it did so by affecting drug uptake or not.

2. Materials and methods

2.1. Materials

Unless otherwise stated, items were obtained from Sigma-Aldrich (Poole, Dorset, UK) or from sources described previously [3]. GSK-3β inhibitor XI (3-(1-(3-Hydroxypropyl)-1H-pyrrolo[2,3-b]pyridin-3-yl]-4-pyrazin-2-yl-
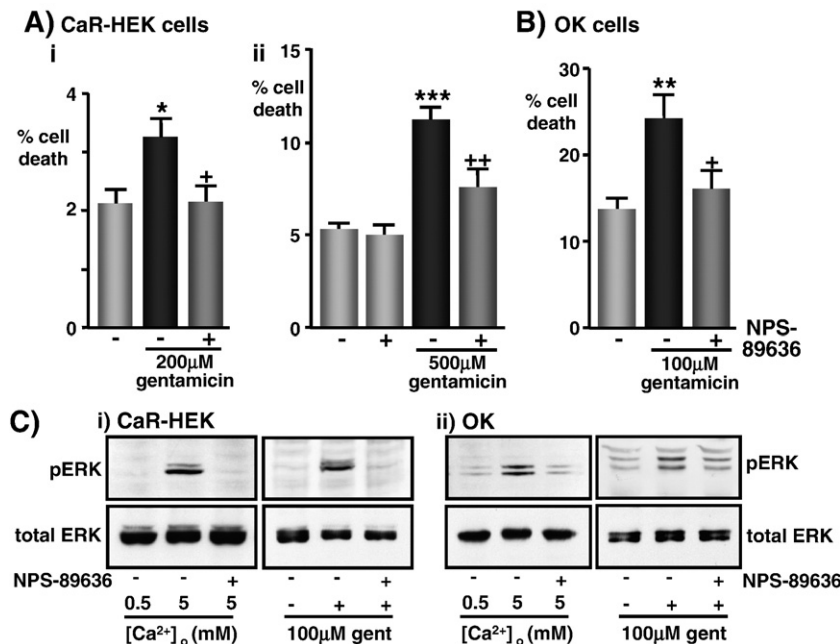

Fig. 2. Cotreatment with the calcilytic NPS-89636 attenuates gentamicin-induced cell death and CaR-induced ERK activation in CaR-HEK and OK cells. CaR-HEK cells were treated for 4 days with 200 (panel Ai, $N=10$) or 500 μM gentamicin (panel Aii, $N=5$) in the presence or absence of 1 μM NPS-89636 (0.01% DMSO vehicle added to controls) and cell death determined by trypan blue exclusion. Alternatively, OK cells were treated for 7 days with or without 100 μM gentamicin in the presence or absence of 500 nM NPS-89636, with addition of fresh serum (1%) to each dish at Day 3 and then cell death quantified as before (panel B, $N=7$). *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control; +$P<0.05$, ++$P<0.01$ vs gentamicin by ANOVA. Panel C, CaR-HEK (i) or OK cells (ii) were stimulated with either 5 mM Ca^{2+}_o, or 100 μM gentamicin in the presence or absence of NPS-89636 (500 nM) for 5 min at 37 °C and then lysed in RIPA buffer as described in Methods. The phospho-ERK content was then determined by immunoblotting using a phospho-specific anti-ERK antiserum and the total levels of ERK shown to be unchanged using an anti-ERK2 polyclonal antibody.
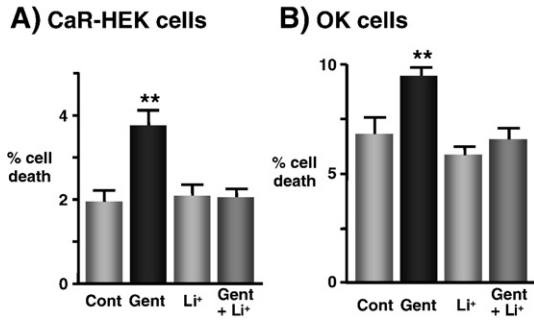
Fig. 3. Cotreatment with lithium ameliorates gentamicin-induced cell death in CaR-HEK and OK cells. CaR-HEK cells were treated for 4 days with 500 μM gentamicin (panel A, N=4–5) in the presence or absence of 1 mM LiCl. Cell death was then determined by trypan blue exclusion. Alternatively OK cells received the same 4 day treatments (panel B, N=8–10). **P<0.01 vs all others, by ANOVA.

pyrrole-2,5-dione) and GSK-3 Inhibitor IX ((2′Z,3′E)-6-Bromoindirubin-3′-oxime) were purchased from Calbiochem.

2.2. Cell Culture

Opossum kidney (OK) cells (used within 12–23 passages of purchase from the American Type Culture Collection, Rockville, MD) and HEK-293 cells, stably transfected with human parathyroid CaR (CaR-HEK) [17], were a gift from Dr. E.F. Nemeth (NPS Pharmaceuticals, Inc., Salt Lake City, Utah, USA) and were cultured as described previously [2,3].

2.3. Cell death counting

Cells (60–70% confluency, 35 mm dishes) were incubated in medium containing 1% serum supplemented with various treatments for up to 4 days, or for up to 7 days with an addition of 1% serum after 3 days to maintain viability. Adherent cells were mixed 1:1 (v/v) with 0.4% Trypan Blue solution. Viable cells (Trypan Blue-excluded) and non-viable cells (Trypan-Blue stained, including cells floating in the medium) were counted on a light microscope using a Neauber's counting slide.

2.4. ERK activation assay

Cells were grown to 80–90% confluence in 35 mm culture dishes and ERK phosphorylation assayed as described previously [2,3]. Briefly, cells were incubated at 37 °C prior to lysis on ice in RIPA buffer supplemented with protease and phosphatase inhibitors and proteins resolved by SDS-PAGE prior to immunoblotting using total ERK and phospho-specific ERK antibodies.

2.5. Flow cytometric DNA analysis

Cells grown in culture flasks were treated as above and then resuspended in serum-free medium containing propidium iodide (10 μg/ml). Flow cytometric analysis was performed using a FACS Vantage flow cytometer equipped with an Enterprise laser (Innova Technology; laser excitation at 488 nm, 250 mV). Red fluorescence (DNA-bound propidium) was detected at 630±22 nm and acquired
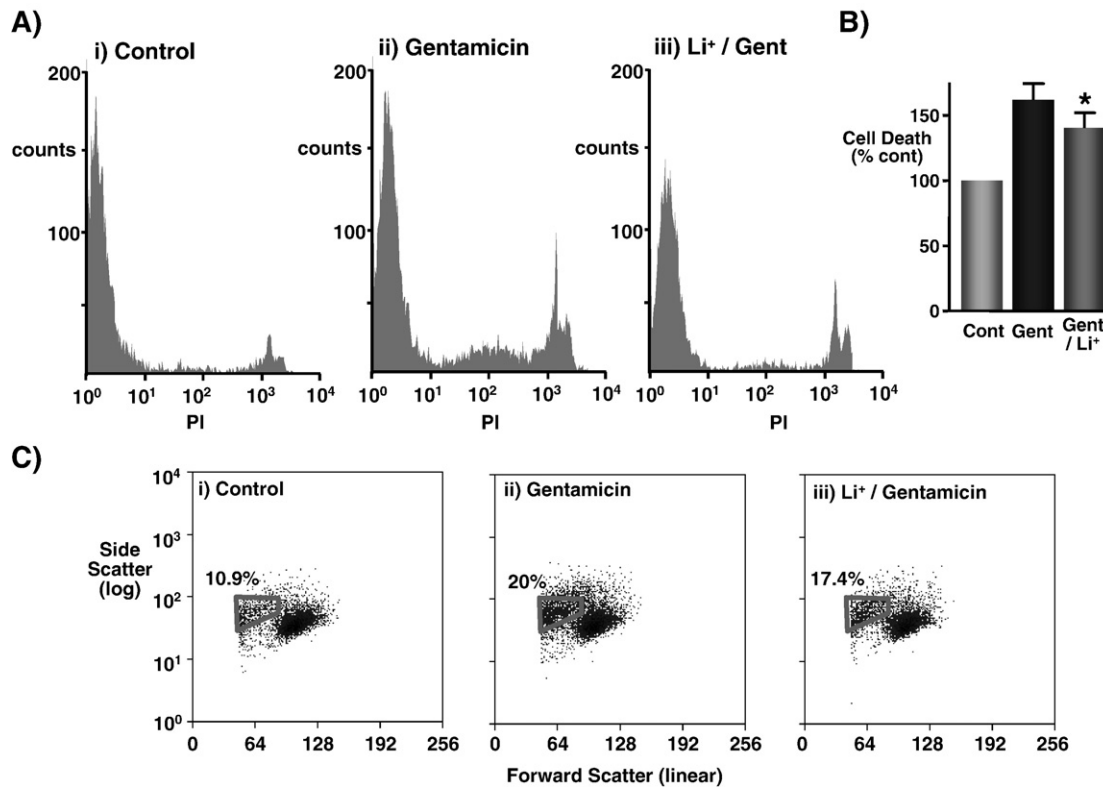

Fig. 4. Flow cytometric analysis of lithium cotreatment on gentamicin-induced CaR-HEK cell death. Panel A, CaR-HEK cells were treated for 4 days in T75 culture flasks with 500 μM gentamicin in the presence or absence of 1 mM LiCl with subsequent cell death assessed by propidium iodide uptake as quantified by flow cytometry (FACS Vantage). Panel B, Histogram showing the pooled cell death data (±s.e.m.) from three independent experiments (including 11 replicates). *P<0.05 gentamicin vs lithium and gentamicin by t-test. Panel C, 2-Dimensional plot showing the cell size and granularity profiles of the cells shown in panel A. In panels A and C, the data shown represent the median responses. Gentamicin increased the proportion of smaller, more granular cells (marked by a polygon and consisting almost exclusively of PI-positive cells) and this effect was ameliorated by LiCl cotreatment.

using logarithmic amplifiers. Forward (FSC) and Side (SSC) light scatter were also recorded to indicate cell size and granularity, respectively. A total of 10,000 cells were analyzed per sample and Cell-Fit software (Becton Dickinson) was used to evaluate the data.

2.6. Texas red gentamicin internalisation

HEK-293 cells, transfected with either pcDNA3.1 or EGFP tagged CaR (CaR-EGFP) [6] vectors were incubated at 37 °C in prewarmed HEPES-buffered physiological saline containing Texas red-conjugated gentamicin (TRG, 1 mg/ml; Molecular Probes, Eugene, OR) for 30 min as described previously [3]. After fixation, the relative levels of TRG uptake were studied by confocal microscopy using an Ultraview confocal optical scanner with a Kr/Ar laser (Perkin Elmer Life Sciences, Cambridge, UK) mounted on an Olympus IX70 inverted microscope. Images were acquired with an Ultrapix CCD digital camera and processed using Perkin Elmer UltraView software package. Laser intensity, shutter speed and image capture speed were constant throughout the acquisition process of each experiment. Quantification of TRG internalisation was performed using a FLUOstar microplate based multi-detection reader (BMG Labtech, Durham, NC, USA).

2.7. Statistical analysis

Unless otherwise stated, data are presented as means ± S.E. and statistical significance determined by one-way ANOVA (Tukey Post-Hoc test; $P < 0.05$).

3. Results

We have shown previously that 4 day exposure of proximal tubule-derived OK cells to 500 μM gentamicin elicits cell death and that increased cell death was observed in CaR-HEK cells following chronic exposure to 200 μM gentamicin [3]. However, the slow evolution of acute renal failure (depression of the GFR) does not normally manifest itself clinically before 5–7 days of treatment, underlying complications notwithstanding [1,18,19]. In addition, the gentamicin concentrations used previously in our cell culture study were an order of magnitude greater than the target plasma concentration aimed for in antibiotic therapy [1]. We therefore examined whether reproducing conditions resembling more closely those achieved therapeutically (i.e., more prolonged exposures and use of lower concentrations of the drug) we could still observe cytotoxicity. Our results show that, indeed, chronic treatment for 6–7 days with 20–100 μM gentamicin elicited significant cell death in CaR-HEK cells (Fig. 1A). Similarly, OK cells exhibited significant cell death when treated with 50 μM gentamicin (Fig. 1B). These dead cells included both floating cells and trypan blue-stained adherent cells.

To demonstrate more conclusively a role for CaR in the development of AGA-induced cellular injury, we used a CaR negative allosteric modulator. Cotreatment with the calcilytic NPS-89636 resulted in a significant reduction in CaR-HEK cell death in response to high concentrations of gentamicin (i.e., 500 μM) and completely ablated the toxic effect of 200 μM when the drugs were administered for 4 days only (Fig. 2A). When OK cells were cotreated with NPS-89636, a significant attenuation (−77%, $P < 0.05$) of gentamicin (100 μM, 7 days)
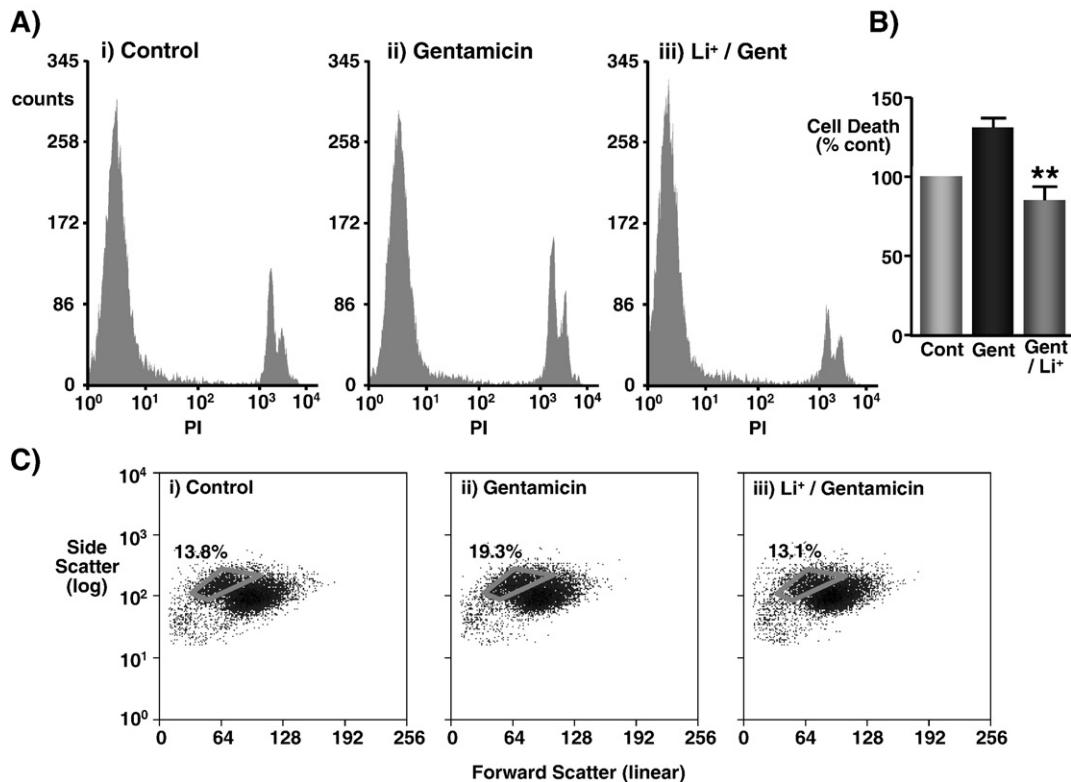

Fig. 5. Flow cytometric analysis of lithium cotreatment on gentamicin-induced OK cell death. OK cells treated for 4 days in T75 culture flasks with 500 μM gentamicin in the presence or absence of 1 mM LiCl were processed for cell death analysis by flow cytometry as described in the Legend to Fig. 4. The gentamicin-induced increase in the proportion of PI-positive cells (panels A and B) and the proportion of smaller, more granular cells (panel C) was attenuated by LiCl cotreatment. **$P < 0.01$ gentamicin vs lithium & gentamicin by t-test ($N = 5$).

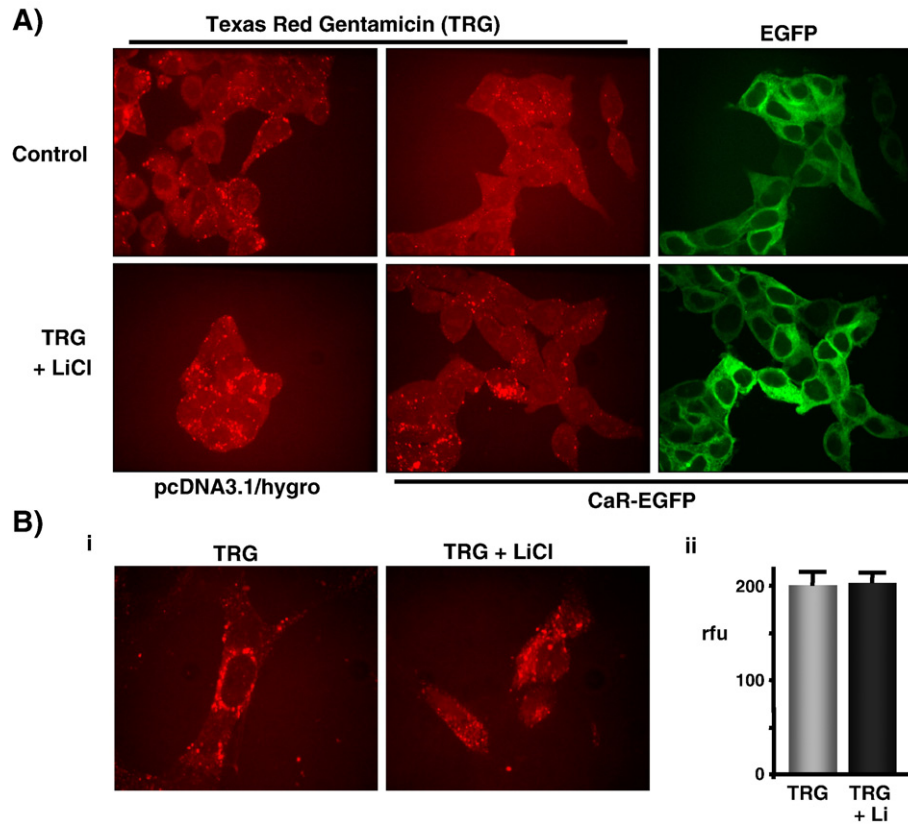　　　*C.E. Gibbons et al. / Biochimica et Biophysica Acta 1782 (2008) 188–195*

Fig. 6. Lithium cotreatment fails to prevent Texas red-Gentamicin uptake into CaR-HEK and OK cells. Panel A, HEK cells, transfected stably with either empty vector (pcDNA3.1/hygro) or human CaR, were preincubated for 10 mins in the presence or absence of 1 mM LiCl and then incubated for 30 min in Texas red-conjugated gentamicin (TRG)±LiCl. Following cell washing to remove excess TRG, microfluorescence imaging revealed equivalent gentamicin uptake in both sets of cells. Panel B, An identical experiment was performed on OK cells. Representative fluorescence images are presented (i) together with a histogram showing relative fluorescence unit (rfu) levels indicative of TRG uptake (ii). Image exposure times were identical for cell fluorescence within each experiment e.g. TRG±lithium. Results are from three independent experiments performed at least in duplicate.

toxicity was observed (Fig. 2B). To confirm that NPS-89636 inhibits CaR-mediated responses in these cells, OK and CaR-HEK cells were stimulated with either 5 mM Ca^{2+}_{o} or 200 μM gentamicin in the presence or absence of the calcilytic and then tested for extracellular-regulated kinase (ERK) phosphorylation / activation, a well-established functional readout for CaR activation [2,20]. Whilst both high Ca^{2+}_{o} concentration and gentamicin elicited ERK activation, as demonstrated previously [2], cotreatment with NPS-89636 blocked these responses in both cell types (Fig. 2C).

There is evidence that lithium may interfere with gentamicin nephrotoxicity in rats [14]. To determine whether lithium exerts its effect directly on renal cells, or via a more systemic effect, we studied gentamicin-induced cell death in both OK cells and CaR-HEK cells in the presence or absence of the clinical target concentration of LiCl (1 mM). Indeed, LiCl cotreatment significantly inhibited the cytotoxicity of gentamicin in CaR-HEK (Fig. 3A) and OK cells (Fig. 3B) as determined by Trypan Blue exclusion. These data were supported by flow cytometry experiments in which lithium cotreatment ameliorated gentamicin-induced cell death in both the CaR-HEK cells (Fig. 4) and OK cells (Fig. 5). In the flow cytometry experiments, the dead cells were those exhibiting increased propidium iodide uptake (Panel A), as well as decreased forward scatter (size) and in-

creased side scatter (granularity; Panel C) indicative of apoptosis [3].

Deterioration of proximal tubular function observed as a consequence of AGA toxicity in patients has been traditionally ascribed to drug endocytosis and its sequestration into lysosomes, with formation of myeloid bodies and phospholipidosis
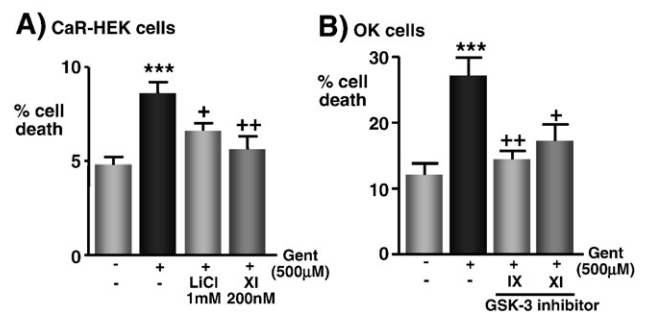

Fig. 7. Inhibitors of glycogen synthase kinase-3 attenuate gentamicin-induced cell death in OK and CaR-HEK cells. Panel A, CaR-HEK cells were treated for 4 days with 500 μM gentamicin in the presence or absence of 1 mM LiCl or 200 nM GSK-3β inhibitor XI and then cell death was then determined by trypan blue exclusion. Panel B, OK cells were treated as in A, but instead cotreated with either GSK-3 inhibitor XI or IX (20 nM). ***$P<0.001$ vs control; +$P<0.05$, ++$P<0.01$ vs gentamicin, by ANOVA; ($N \geq 7$).

[22]. To examine whether the apparently protective effect of lithium occurs by inhibiting gentamicin internalisation, we employed a texas red-conjugated form of gentamicin (TRG) [3]. Interestingly, lithium did not appear to reduce gentamicin cellular uptake. Confocal images of CaR-EGFP-transfected HEK cells (Fig. 6A) and OK cells (Fig. 6B) pretreated with LiCl (1 mM, 30 min) and then incubated in TRG (+1 mM LiCl, 30 min, 37 °C) showed similar levels of TRG uptake compared to that observed in non-lithium treated cells. These observations were reproduced using a quantitative fluorometric assay (OK cells, $P > 0.05$) (Fig. 6Bii).

Finally, lithium is a known inhibitor for glycogen synthase kinase (GSK)-3β and inhibition of this kinase could contribute to the apparent cell-protective effect of lithium [23]. Therefore, we investigated whether GSK-3β inhibition is capable of attenuating gentamicin-induced cell death in OK and CaR-HEK cells. Indeed, the GSK-3β inhibitor XI inhibited (500 μM) gentamicin-induced cell death in CaR-HEK (Fig. 7A) and OK (Fig. 7B) cells, whilst the GSK-3 Inhibitor IX also inhibited gentamicin toxicity in the OK cells (Fig. 7B).

4. Discussion

Here we provide pharmacological evidence that the CaR is capable of mediating AGA-induced toxicity in cultured proximal tubule-derived cells and that such cellular toxicity can be ameliorated by lithium cotreatment. The CaR negative allosteric modulator (calcilytic) NPS-89636 has been shown previously to inhibit high $[Ca^{2+}]_o$-induced mineralisation in fetal rat calvarial cells [24], and in CaR-HEK cells to inhibit calcimimetic-induced actin polymerisation [25] and CaR_{T888} phosphorylation [26]. Other calcilytics have been shown to stimulate PTH secretion in rats and isolated bovine parathyroid cells [27–29] as well as inhibiting inositol phosphate metabolism and Ca^{2+}_i mobilisation in CaR-HEK cells [27,29,30]. Here we demonstrate that NPS-89636 inhibits acute high $[Ca^{2+}]_o$- and gentamicin-induced ERK activation and chronic gentamicin-induced cell death in OK and CaR-HEK cells. Initially, 1 μM NPS-89636 was employed (Fig. 2A) as was used previously [24–26] however in later experiments (Fig. 2B and C) 500 nM was found to be equally effective. The simplest explanation for these data is that NPS-89636 is acting by inhibiting CaR activity, presumably by decreasing receptor agonist sensitivity. This observation is supported by our previous studies in which we have demonstrated CaR expression and function in OK cells [2] and shown [3] that OK cell death can also be induced by the CaR agonists spermine [31] and poly-arginine [32]. Furthermore, we have shown previously that using similar gentamicin concentrations, the drug elicits significant apoptotic cell death in CaR-HEK cells but not in HEK cells stably transfected with the empty vector alone [3]. Thus, it would be interesting to determine whether calcilytic cotreatment protects gentamicin-injected rats from proximal tubule injury.

Variations in basal cell death levels between experiments were observed. The precise amount of basal cell death recorded in any experiment depends on a variety of factors including the rate of growth of the cells, the depletion of nutrients, space and growth factors, as well as the method by which cells are collected and cell death quantified. In general however, we found both here and previously (3) that the relative toxic effect of gentamicin tended to be quite consistent. With regards the gentamicin exposure conditions it should be noted that the drug was present in the media throughout the 4–6 day treatments whereas with clinical use, daily peaks and troughs in plasma gentamicin concentration would alter the pharmacodynamics of proximal tubular exposure to the drug. The possible consequence, if any, of such a difference is unclear and thus an in vivo study will be necessary to confirm the significance of the cell study.

We also demonstrated a protective effect of lithium on gentamicin-induced cell death in the renal-derived cells. The effect was significant though partial in some experiments (Figs. 4 and 7), but complete in others (Figs. 3 and 5). Whether higher concentrations of lithium would have consistently ablated gentamicin-induced apoptosis was not tested. The 1 mM lithium treatment used here was chosen deliberately to correspond to the target therapeutic plasma concentration employed in bipolar disorders. Since alternative theories of gentamicin nephrotoxicity involve AGA endocytosis [18], then one possible explanation for the apparent protective effect of lithium would be that it attenuates gentamicin cellular uptake by interfering with the turnover of negatively-charged inositol phospholipids with which gentamicin would bind on the membrane. However, at the gentamicin concentration tested, lithium failed to reduce the acute cellular uptake of Texas red-conjugated gentamicin into the CaR-HEK or OK cells, despite decreasing the chronic toxicity of the AGA. Therefore, since lithium can also interfere in the glycogen synthase kinase-mediated pathway, which is involved in the regulation of cell fate [23], we tested the effect of GSK-3β inhibitors and found that they also inhibited gentamicin-induced CaR-HEK and OK cell toxicity. Our observations implicate GSK-3β signalling in the mediation of the CaR-mediated gentamicin toxicity seen here, possibly via the GSK3/Wnt/β-catenin pathway. Whilst this does not prove that the protective effect of lithium occurs via GSK-3β inhibition, there at least appears to be good correlation between the protective effects of lithium and of the GSK-3β inhibitor XI. In the brain, the neuroprotective effect of lithium treatment has been associated with altered expression of pro-and anti-apoptotic proteins such as BAX and Bcl-2 respectively [33] and this may play a role here. In any case, we propose that lithium should now be tested in rats to determine whether it may serve as an effective ameliorant for AGA-induced nephrotoxicity. In this regard, lithium has already been shown to reduce the formation of lysosomal myeloid bodies in rat renal cortex following chronic gentamicin treatment [14]. It should be noted that while we have shown that lithium fails to reduce total TRG uptake into the cells while attenuating gentamicin toxicity, we cannot rule out the possibility that lithium may divert the gentamicin from one intracellular locale to another one in which the AGA exerts reduced toxicity. In this regard, others have shown that porcine, proximal tubular-derived LLC-PK1 cells traffic TRG rapidly to the endoplasmic reticulum and then on to the cytosol and nucleus [34] and thus lithium could act by interfering with such a process.

Previous studies have shown that AGA-elicited cellular events include the impairment of glucose, protein and ion transport [18] as well as numerous biochemical abnormalities [18,35]. Since gentamicin elicits OK cell toxicity over a similar 6-day time course to the appearance of nephrotoxicity in humans and at concentrations as low as 50 μM, we would propose that future cellular studies of gentamicin toxicity should use concentrations of drug much closer to the target plasma concentration in humans [1]. At high concentrations, the AGAs exhibit non-cell specific cytotoxicity by interfering with protein translation and are indeed often employed as selection agents in mammalian expression systems. Therefore, high gentamicin concentrations may induce cytotoxicity by means not related to the clinical nephrotoxic mechanism, especially given that gentamicin is not generally cytotoxic in other organs (except for its ototoxicity) during clinical therapy. In an elegant series of experiments, El Mouedden et al. demonstrated that rats receiving low dose gentamicin treatment, i.e. at low multiples of the therapeutic dose, exhibit proximal tubule cell apoptosis whereas higher doses of drug were required to elicit acute tubular necrosis [36]. Accordingly, low concentrations of gentamicin, as used in the current study, may elicit different toxic responses and by alternate cellular mechanisms than for higher doses and thus may have greater relevance in understanding the proximal tubular toxicity of the AGAs.

The use of AGAs with lower nephrotoxic potential, such as amikacin, isepamacin or even the C2 gentamicin congener [37] in addition to the use of single daily dosing will lead to a reduction in the prevalence of AGA nephrotoxicity where employed. However due to its low cost, gentamicin therapy continues to be widely-used globally and thus it remains important to understand the molecular cause(s) of the proximal tubule injury particularly if an economical cotreatment adjunct such as lithium can be shown to be an effective ameliorant. In this regard, it will be necessary next to show that lithium actually ameliorates AGA-induced nephrotoxicity in vivo, for example in rats.

The physiological function(s) of the proximal tubular CaR may include the regulation of volume absorption, vitamin D 1α-hydroxylation and phosphate reabsorption [21,38–40]. We propose therefore that during AGA therapy the presence of luminal gentamicin may cause additional and excessive activation of the proximal tubular CaR causing sustained receptor-induced signalling leading to apoptotic cell death and that this should now be tested in an animal model. Together, these data implicate the CaR and a lithium-inhibitable signalling pathway in the mediation of gentamicin toxicity in proximal tubular-derived OK cells and CaR-HEK cells.

Acknowledgments

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